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Apoptosis-Related Factors p53, Bcl_2, and Bax in Neuroendocrine Lung Tumors

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Neuroendocrine (NE) lung tumors comprise four classes of progressive aggressiveness for which proliferation and apoptosis rates could both contribute to their distinctive behavior. As p53 mutations may favor escape from apoptosis through changes in Bcl_2-Bax expression balance, which are survival and apoptotic genes, respectively, we studied 121 NE lung tumors (16 typical carcinoids (TC), 5 atypical carcinoids (AC), 29 large-cell NE carcinomas (LCNECs), and 71 small-cell lung carcinomas (SCLCs) using immunohistochemistry. We quantified apoptosis by terminal-deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) in 31 of these cases. There was a significant increase of p53 mutant immunophenotype (defined as immunoreactivity with at least two antibodies for at least 20% of tumor cells) between atypical/typical carcinoids group and the LCNEC/SCLC group (P = 0.0003). There was an inverse correlation (P < 0.0001) between the scores of Bax and Bcl_2 expression in individual tumors and a significant inversion of the Bcl_2:Bax ratio between low-grade (typical and atypical carcinoids) and high-grade (LCNECs and SCLCs) tumors with a predominant Bax expression in the first group and predominant Bcl_2 expression in the second. Whereas carcinoids had variable apoptotic indexes, LCNECs had high indexes (1.3 to 6.8%), and SCLCs had almost no apoptosis (<0.1%). Bcl_2 overexpression, Bax down-regulation, and Bcl_2:Bax ratio >1 correlated with lower apoptotic index in both LCNEC and the pool of LCNECs and SCLCs (P < 0.05) and a lower survival rate in the group of atypical and typical carcinoids and LCNECs (P < 0.002). The highest levels of Bcl_2 expression and Bcl_2:Bax ratios were associated with p53 mutant immunophenotype (P = 0.02). Our results suggest that aggressiveness in NE lung tumors could be linked, in addition to proliferation, to apoptosis-related factors. (Am J Pathol 1996, 149:1941–1952)

Lung neuroendocrine (NE) tumors include a spectrum of four clinicopathological entities with varying degrees of aggressiveness. Typical carcinoids (TCs) are lower-grade, well differentiated NE tumors with excellent prognosis (nearly 100% 10-year survival). Atypical carcinoids (ACs) keep morphological NE characteristics but show some cellular atypia, loci of necrosis, and mitotic activity (3 to 10 mitoses per 10 high-power fields).[1] High-grade NE lung tumors comprise large-cell NE carcinoma (LCNEC)[2] with neuroendocrine morphology but marked cellular pleiomorphism, prominent necrosis, and high mitotic activity (≥20 mitoses per 10 high-power fields). Small-cell lung carcinoma (SCLC) and LCNEC differ essentially by cell size and chromatin morphology. The two intermediate categories (AC and LCNEC) have a more unpredictable prognosis than TC and SCLC, and alternative markers are needed to assist classification and prognosis. A few studies have focused on the genetic alterations on the spectrum of NE lung tumors.

Clonal expansion and tumor growth is the result of the deregulation of intrinsic proliferation (cell division) and cell death (apoptosis).[3,4] Among the multiple genetic events associated with the development of lung cancer,[5] and especially NE lung cancers, are activation of dominant proto-oncogenes such as *myc* family oncogenes[6–9] and inactivation of recessive tumor suppressor genes associ-

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ated with chromosomal deletion such as the retinoblastoma gene *Rb*[10,11] and *p53*. Loss of these tumor suppressor functions can actively promote proliferation. *p53* genetic alterations leading to aberrant protein stabilization in the cell nucleus have been detected using imunohistochemistry in approximately 50% of non-SCLCs[12,13] and in 70 to 100% of SCLCs using sequence analysis (single-strand conformation polymorphism or complete sequencing).[14–16] *p53* genetic alterations are detected in SCLCs and non-NE lung cancer (non-SCLCs) with similar frequencies.

The *bcl*$_2$ gene was originally discovered in a follicular B-cell lymphoma where a chromosomal translocation t(14;18) moves the *bcl*$_2$ gene into juxtaposition with transcriptional enhancer elements of the immunoglobulin heavy chain locus.[17] In contrast, transregulatory mechanisms appear to be responsible for the high levels of Bcl_2 protein production that occurs in many different types of solid tumors such as prostate,[18] thyroid,[19] breast,[20] neuroblastoma,[21] and non-SCLCs.[22,23] One of the potential transregulators of *bcl*$_2$ in cancer is the tumor suppressor protein p53. Negative responsive elements for wild-type p53, but not mutant p53, have been found in the 5′ untranslated regions of the *bcl*$_2$ gene through which wild-type but not mutant p53 is able to mediate repression of the *bcl*$_2$ gene.[24] p53 mutations could thus result in elevated production of Bcl_2 at least in some tissues. In addition, in co-transfection assays, wild-type p53, but not mutant p53, was found to strongly transactivate the expression of a homologue of *bcl*$_2$ termed *bax*, and a p53 binding site was identified in the *bax* gene promoter.[25,26] The Bax protein, sharing 21% homology with Bcl_2 protein, can be considered as the main effector of apoptosis.[27] Its function in active cell death as a dimer Bax-Bax can be opposed by heterodimerization with Bcl_2 (Bax-Bcl_2). Thus, p53 appears to be the main regulator of apoptosis through regulation of the Bax-Bcl_2 balance, and p53 mutation, or any other type of p53 inactivation, could be responsible for abrogation of cell death through Bax-Bcl_2 imbalance.

In the present paper we investigated the levels of expression of the Bax and Bcl_2 proteins in the spectrum of NE lung tumors to correlate their expression to the immunohistochemical status of the *p53* gene. We found that Bax and Bcl_2 were opposed in their expression in the NE tumor cells and that each different tumor type had a characteristic profile of Bcl_2-Bax expression that was statistically inverse in carcinoids *versus* highly aggressive LCNECs and SCLCs. We found that Bcl_2-Bax balance was partially dependent on p53 and correlated more with clinicopathological parameters than did p53 immunohistochemical status itself.

Table 1. *Histological Type and Stage of the 121 NE Lung Tumors Studied*

	n	Stage of primary tumors		Metastases*
		I and II	III and IV	
TC	16	15	1	0
AC	5	3	2	0
LCNEC	29	12	17	12
SCLC	71	4	67	61

*Number of node metastases studied instead of primary tumors.

Materials and Methods

Tumor Samples

The histological distribution of the 121 lung NE tumor samples and their clinical stage are shown in Table 1. For obvious clinical reasons, TC and AC samples were obtained at surgical resection as these tumors are always surgically treated, whereas most SCLC and some of the LCNEC samples were obtained at a lymph node metastatic location by biopsy through mediastinoscopy. Samples were immediately frozen at surgery and kept at −80°C until study. They were then fixed in 10% formalin and/or alcoholic Bouin's fixative for histological classification and staging and immunoanalysis.

Immunohistochemical Analysis of p53, Bcl_2, and Bax

p53, Bcl_2 and Bax immunostaining was performed on frozen sections fixed with 4% paraformaldehyde using classical immunoperoxidase techniques. Three p53 monoclonal antibodies were used: DO-7 (1/50; Dako, Glostrup, Denmark), PAb-1801 (1/300; Oncogene Science, Uniondale, NY), and PAb-240 (1/300; Oncogene Science). Bcl_2 was detected with the monoclonal antibody from Dako (1/100) and Bax with the N-19 rabbit polyclonal antibody (1/200; Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation at 4°C with the primary antibody, slides were washed in phosphate-buffered saline (PBS) and then exposed to the secondary antibody, biotinylated donkey $F(ab')_2$ anti-rabbit (1/1000; Jackson Laboratories, West Grove, PA) or anti-mouse (1/400; Jackson), for 1 hour at room temperature. They were then washed in PBS and incubated with the streptavidin-biotin-peroxidase complex (1/400; Dako) for 1 hour at room temperature.

The chromogenic substrate of peroxidase was a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride/0.03% H_2O_2/10 mmol/L imidazole in 0.05 mol/L Tris buffer, pH 7.6. The slides were counterstained with Harris' hematoxylin. Normal rabbit or mouse IgG at the same concentration as the primary antibodies served as negative controls.

Results were recorded by two investigators independently assessing the percentage of positive cells and the intensity of staining. For Bcl_2 and Bax, scores of immunostaining were calculated by multiplying the percentage of labeled cells with the intensity (1+, 2+, or 3+) of staining as compared with the 3+ Bcl_2 intensity of the background lymphocytes.

Quantification of Apoptotic Cells

Thirty-one lung NE tumors (six TCs, thirteen LCNECs, and twelve SCLCs) of our series were analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL). These paraffin-embedded samples had all been fixed with 4% formalin, a standardized approach necessary for comparing TUNEL-based apoptotic indexes.[28]

Briefly, the *in situ* cell detection kit (Boehringer Mannheim, Mannheim, Germany), which contains calf thymus TdT, fluorescein-dUTP and a peroxidase-labeled anti-fluorescein sheep Fab fragment, was used according to the manufacturer's instructions improved by a preincubation with 20% normal bovine serum (Jackson) for 30 minutes at room temperature before the TdT-dUTP step (1 hour at 37°C) and a resaturation (30 minutes at room temperature) with 20% normal sheep serum (Jackson) in 1% (w/v) blocking reagent (Boehringer Mannheim) in 0.1 mol/L Tris-buffered saline before the antibody step (30 minutes at 37°C). After the diaminobenzidine color reaction, counterstaining was performed with Harris' hematoxylin. A negative control was performed by omitting TdT from the labeling mix.

TUNEL results were quantified using the Glasgow cell-counting graticule[29] (Datasights, Enfield, UK). The apoptotic index (AI) was expressed as the percentage of positive nuclei for at least 5000 counted cells.

The 12 SCLCs were also double labeled for TUNEL and Bcl_2 (data not shown). Double labelings were performed in a sequential manner, with the Bcl_2 immunostaining as the first step using a streptavidin-biotin-alkaline phosphatase complex (1/100; Dako) revealed with Fast Red (Sigma-Aldrich, St. Quentin Fallavier, France).

Table 2. *p53 Immunoreactivity in 121 Lung NE Tumors According to Histological Type*

	n	p53-positive immunostaining	*P**
TC	16	0	
			NS
AC	5	1	
LCNEC	29	17	
			NS
SCLC	71	34	
TC plus AC	21	1	
			0.0003
LCNEC	29	17	
TC plus AC	21	1	
			0.0003
SCLC	71	34	

p53 was considered as positive when at least 20% of cells were stained with two antibodies.
*Fisher exact test.

Statistical Analyses

Differences between independent groups were determined by means of the Kruskal-Wallis H test and the Mann-Whitney *U* test. Differences between proportions were evaluated using the Fisher exact test. Actuarial survival rates were calculated from the time of treatment until the 1st of January 1996 using the method of Kaplan and Meier. The comparison of survival was performed using the log-rank Mantel-Cox test. A *P* value less than 0.05 was considered significant.

Results

p53 Immunostaining

Results are shown in Table 2 and Figure 1, A and B.

p53 immunostaining (Figure 1, A and B) was considered as positive when at least 20% of cells had stained nuclei with at least two of the three antibodies used. This high cut-off was inferred from previous comparison of a complete sequence analysis of the p53 gene and immunohistochemical staining in 20 cases of NE and non-NE lung carcinoma.[13] Positive immunostaining so defined was 100% concordant with a missense mutation in or outside highly conserved exons 5 to 8 and was termed mutant immunophenotype. Using this cut-off, p53-positive immunostaining could be considered as indicative of mutant p53 (mutant immunophenotype) in lung cancers. This of course does not imply that negative staining was indicative of functional (wild-type) p53, as in the same study 20% of false negatives (mutant p53 with null protein phenotype) were described, as further discussed. In some tumors of the present study, scattered positive cells (less than 20%) were

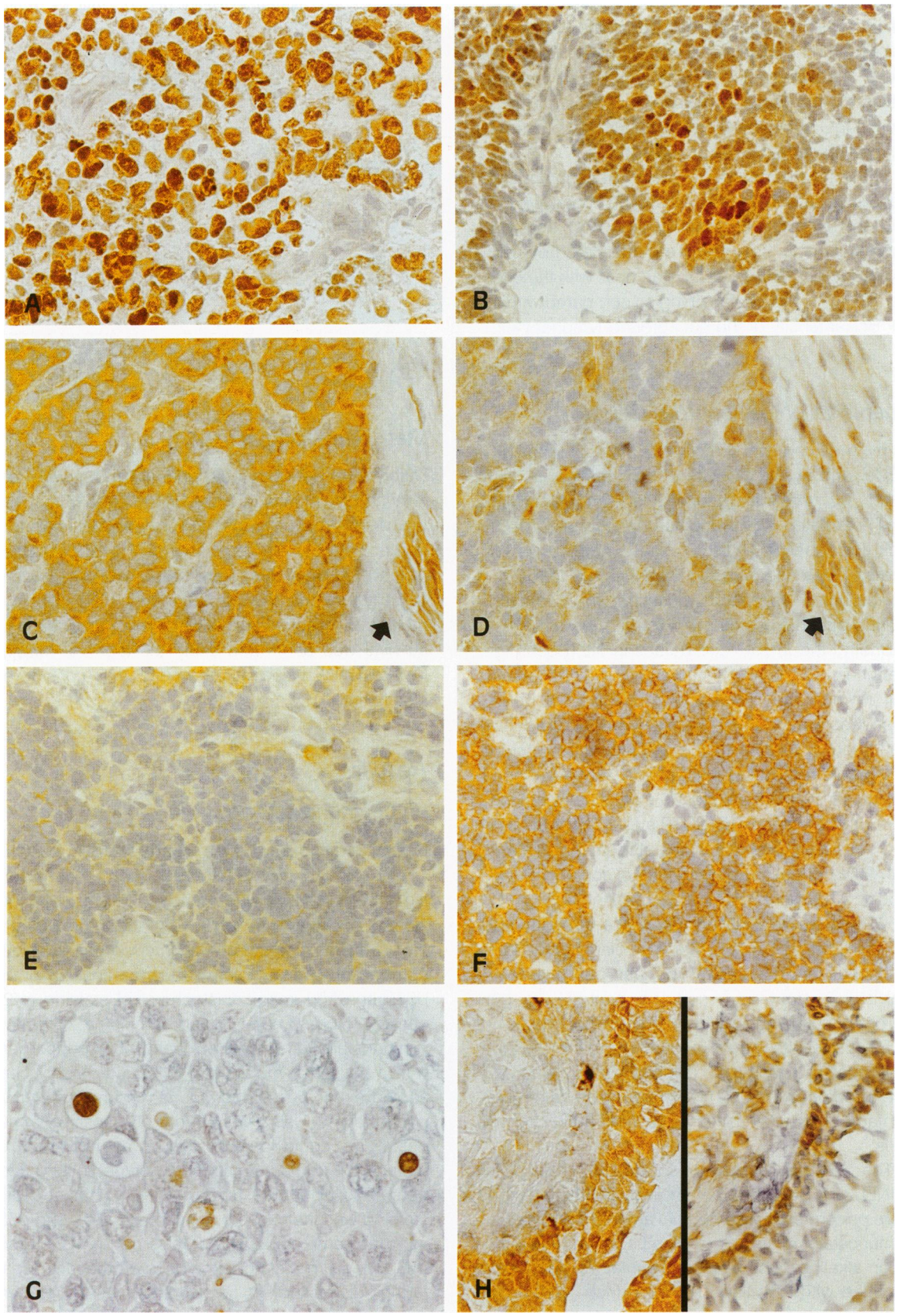
A
B
C
D
E
F
G
H

Table 3. *Bcl_2 and Bax Immunoreactivity in 121 Lung NE Tumors*

A. Bcl_2 and Bax reciprocal pattern of expression			
	$Bcl_2 \leq 50$	$Bcl_2 > 50$	P^*
Bax ≤ 50	6	52	0.0011
Bax > 50	23	40	

B. Bcl_2 and Bax immunoreactivity according to histological type				
	n	$Bcl_2 > 50$	Bax > 50	P^*
TC	16	3	15	
AC	5	0	4	
LCNEC	29	26	10	
SCLC	71	64	34	
		$Bcl_2 \leq 50$	$Bcl_2 > 50$	
TC plus AC	21	18	3	<0.0001
LCNEC plus SCLC	100	10	90	
		Bax≤50	Bax>50	
TC plus AC	21	2	19	<0.0001
LCNEC plus SCLC	100	56	44	

C. Mean level of Bcl_2 and Bax expression according to histological type					
	n	Bcl_2 mean score ± SD	$P^\dagger$	Bax mean score ± SD	$P^\dagger$
TC	16	43 ± 76	NS	182 ± 67	NS
AC	5	10 ± 22		160 ± 114	
LCNEC	29	191 ± 99	NS	60 ± 78	NS
SCLC	71	209 ± 99		75 ± 78	
TC plus AC	21	35 ± 68	<0.0001	177 ± 79	<0.0001
LCNEC plus SCLC	100	204 ± 99		70 ± 78	

Bcl_2 and Bax scores of expression = percentage of stained cells × intensity (1 to 3). NS, not significant.
*Fisher exact test.
†Mann-Whitney *U* test.

seen with one or two antibodies (2 of 16 TCs, 2 of 29 LCNECs, and 6 of 71 SCLCs; these 10 cases were not considered as p53 positive). For all other cases, a few discrepancies were seen between different antibody reactivities in the same tumor, although PAB 240 was less reactive and gave negative staining in otherwise positive cases (PAB 1801 and DO7 positive in at least 20% of cells) in 1 AC, 1 LCNEC, and 3 SCLCs.

p53 immunoreactivity (mutant immunophenotype) was never found in TCs, rarely in ACs, and in approximately 50% of LCNECs and SCLCs. There was a significant increase of p53 mutant immunophenotype between carcinoids as a group (TCs and ACs) and each of the highly aggressive NE tumors, LCNECs and SCLCs ($P = 0.0003$), which displayed the same frequency of p53 alterations.

Bcl_2 and Bax Immunostaining

The results are shown in Table 3 and Figure 1, C–F.

Immunoblotting (Western) was performed in parallel in 20 cases with the same antibodies (data not shown) and indicated the expected p26 protein for

Figure 1. A: *p53 immunostaining with antibody PAB 1801 in a LCNEC with 90% of cell nuclei positive and no positive stromal cells. Magnification, ×200.* B: *p53 immunostaining with antibody DO7 in a SCLC with 50% of cell nuclei positively stained. Magnification, ×200.* C *and* D: *Bax cytoplasmic immunostaining in a carcinoid* (C) *where Bcl_2 immunostaining is negative despite highly reactive stromal lymphocytes and endothelial cells* (D). *Note a nerve, positive with both antibodies* (arrows). *Magnification, ×200.* E *and* F: *SCLC negative for Bax* (E) *and positive for Bcl_2* (F). *Magnification, ×200).* G: *TUNEL-stained apoptotic cells in a LCNEC. Magnification, ×200.* H, left: *Bax immunostaining of normal bronchial epithelium. All epithelial cells are positively stained.* H, right: *Bcl_2 immunostaining on the same bronchiole. Specific staining is restricted to the basal cells. Magnification, ×200.*

Bcl_2 and p21 protein for Bax. The p24-Bax-β isoform was not detected. Immunoblots were found in close correlation with immunohistochemistry in 70% of the cases. In the other 30%, discrepancies may be attributed to variable dilution of tumor cells by the stromal cells and, for Bcl_2, to expression in lymphoid cells in node metastases that probably obscured the signal from tumor cells. Thus, immunohistochemistry was preferred to Western blotting in the present study.

Bcl_2 and Bax immunostaining was detected in the cytoplasm with finely granular pattern (Figure 1, C–F). Reciprocal patterns of Bax and Bcl_2 protein production were observed in individual tumors ($P = 0.0011$, Table 3A). The majority of carcinoids (TCs and ACs; 20/21) showed a Bcl_2:Bax ratio ≤ 1, whereas the majority of LCNECs and SCLCs (90/100) had a high expression of Bcl_2 and a low Bax expression (Bcl_2:Bax ratio > 1). As a consequence, there was a significant inversion of the Bcl_2:Bax ratio between carcinoids (TCs and ACs) and LCNECs and SCLCs ($P = 0.0016$), the two last classes being identical. The frequency of Bcl_2 overexpression (score > 50) and the mean score of expression were both significantly lower in carcinoids (TCs and ACs) on the one side and LCNECs and SCLCs on the other side ($P < 0.0001$). Inversely, the frequency of Bax overexpression (score > 50) and mean score of expression were significantly higher in carcinoids (TCs and ACs) than in LCNECs and SCLCs ($P < 0.0001$ for both; Table 3, B and C).

Of interest, on normal bronchioli surrounding tumors, Bcl_2 was present in basal cells (Figure 1H) and also in some elongated rare cells extending from basal layer to lumina, with distribution and location suggestive of normal NE cells.

Bcl_2-Bax Expression in Treated versus *Untreated SCLC Patients*

In 15 cases for which pretreatment and post-therapy samples were available, we observed a marked increase of Bcl_2 expression after treatment. Where the staining was mild and heterogeneous (intensity of 1+ or 2+ on 30 to 80% of tumor cells) before therapy, it was diffuse (80 to 100%) and intense (3+) on the post-therapy sample. This increase was observed in 12 of 15 (80%) of patients treated for SCLC. In contrast, there were no clear differences in the Bax immunostaining.

Table 4. *Bcl_2 and Bax Expression and Their Ratio According to p53 Immunophenotype in 121 Lung NE Tumors*

	p53-positive immunostaining	p53-negative immunostaining	*P**
$Bcl_2 \leq 50$	6 (21%)	23	
			0.005
$Bcl_2 > 50$	47 (51%)	45	
Bax ≤ 50	32 (55%)	26	
			0.018
Bax > 50	21 (33%)	42	
Bcl_2:Bax ≤ 1	6 (23%)	20	
			0.024
Bcl_2:Bax > 1	47 (49%)	48	

p53 was considered as positive when at least 20% of cells were stained with two antibodies. Bcl_2 and Bax scores of expression (≤ 50 or >50) = percentage of stained cells $\times$ intensity (1 to 3).

*Fisher exact test.

Bcl_2-Bax Expression in Relation to p53 Immunophenotype

Bcl_2 and Bax expression was compared in patients with or without mutant immunophenotype (Table 4) in the entire spectrum of NE tumors. Of 92 NE tumors with high Bcl_2 expression (score > 50), 51% showed p53 mutant immunophenotype, whereas of 29 NE tumors with low Bcl_2 expression (score ≤ 50), 21% showed p53 mutant immunophenotype ($P = 0.005$). Inversely, of 63 tumors with high Bax expression, 33% showed p53 mutant immunophenotype, whereas of 58 tumors with low Bax expression, 55% showed p53 mutant immunophenotype ($P = 0.018$). Finally, of 95 NE tumors in which Bcl_2 was predominantly expressed (Bcl_2:Bax ratio > 1), 49% showed p53 mutant immunophenotype, whereas of 26 NE tumors in which Bax was predominantly expressed, 23% showed p53 mutant immunophenotype ($P = 0.024$). Characteristic profiles for p53, Bax, and Bcl_2 expression could be discerned between carcinoids (TCs and ACs), with Bax expression predominant over Bcl_2 and no p53 mutant immunophenotype, and the highly aggressive LCNECs and SCLCs, with Bcl_2 predominant over Bax and a high frequency (~50%) of mutant immunophenotype. Overall, p53 mutant immunophenotype, across the histiotypes, influenced Bcl_2 toward high expression, Bax toward low expression, and Bcl_2-Bax balance to become greater than unity.

Analysis of Apoptosis by TUNEL in Lung NE Tumors

Results are summarized in Table 5.

ACs were not examined because no formalin-fixed sample was available. All lung NE tumors examined

Table 5. *Apoptotic Indices and p53, Bcl_2, and Bax Immunoreactivity in 31 Lung NE Tumors*

Histological type	AI	AI rank average (P = 0.0002)*	p53	Bcl_2 score	Bax score	Bcl_2:Bax
TC	<0.1	11.7	−	0	160	<1
	<0.1		−	0	160	<1
	<0.1		−	50	160	<1
	1.2		−	0	200	<1
	1.8		−	50	80	<1
	10		−	100	200	<1
LCNEC	1.3	23.8	+	300	0	>1
	1.8		−	210	0	>1
	1.9		+	300	10	>1
	1.9		+	300	0	>1
	2.6		−	300	0	>1
	2.7		−	300	100	>1
	3.3		+	0	20	<1
	3.3		+	200	0	>1
	3.5		+	160	15	>1
	3.7		−	0	160	<1
	3.8		+	240	0	>1
	4		+	150	150	1
	6.8		−	0	300	<1
SCLC	<0.1	9.7	−	300	0	>1
	<0.1		−	160	20	>1
	<0.1		−	240	40	>1
	<0.1		−	300	0	>1
	<0.1		+	0	100	<1
	<0.1		+	300	0	>1
	<0.1		+	160	20	>1
	<0.1		+	300	0	>1
	1		+	240	0	>1
	1.1		−	270	240	>1
	1.1		+	300	100	>1
	1.8		−	300	100	>1

p53 was considered as positive when at least 20% of cells were stained with two antibodies. Score = percentage of stained cells × intensity (1 to 3).
*Kruskal-Wallis H test.

showed a proportion of TUNEL-stained cells, lying free in the tissue or as phagocytosed apoptotic bodies. The apoptotic cells were isolated but not randomly distributed, their density being conspicuously different from one area to another within the same sample. Most of these cells exhibited apoptotic morphology, containing strongly labeled condensed nuclei and micronuclei, with signal reinforcement at the nuclear border. Labeled, morphologically normal cells were generally few and were counted only when the labeling was very intense and the background absent. Necrotic areas were unlabeled but sometimes surrounded by rims of apoptotic cells; these were avoided in quantification. Mitoses were not labeled. Lack of TdT in the TUNEL mix completely abolished labeling.

As shown in Table 5, LCNECs had uniformly high apoptotic indexes (AIs; Figure 1G), reaching as much as 6.8% of the cellular population in one case, whereas SCLCs showed rare, scattered labeled cells. TCs appeared as a heterogeneous class having widely divergent AIs (<0.1 to 10%).

No correlation between AI and p53 immunoreactivity, Bcl_2 or Bax scores, or Bcl_2:Bax score ratios could be shown across the histological groups (ie, in the pool TCs plus LCNECs plus SCLCs). Despite their wide range of AIs, the six TCs we included in the TUNEL analysis were homogeneously negative for p53, had all Bax scores >50, Bcl_2 scores ≤50 (with one exception at 100), and their Bcl_2:Bax ratios less than unity. This presumably explains the lack of correlation between AI and immunoreactivity scores for p53, Bcl_2, Bax, and the Bcl_2:Bax ratio for TCs taken singly. For LCNECs, Bax scores >50 and Bcl_2:Bax ratios ≤1 corresponded to higher AIs (Mann-Whitney U test, P = 0.0443 and 0.0249, respectively). Bax scores >50 correlated with higher AIs in the SCLC subgroup (P = 0.0198) as did Bcl_2:Bax ratios ≤1 in the pool LCNECs plus SCLCs (P = 0.0381). Probably due to the limited number of cases, no other immunophenotype-TUNEL correlation reached statistical significance. However, in SCLCs (Table 5), the lack of inverse correlation between AIs and the high Bcl_2 scores and Bcl_2:Bax score ratios could be

the result of the extreme rarity of TUNEL-positive cells, which precluded differential measure of the index. Therefore, we performed Bcl_2-TUNEL double labelings in 12 SCLC cases (data not shown), which indicated a clear-cut separation of tumor areas containing TUNEL-positive apoptotic cells from those expressing Bcl_2, which is in agreement with the anti-apoptotic function of the Bcl_2 protein.

Bcl_2-Bax Expressions in Relation to Myc Family Gene Status

When Bcl_2 or Bax expressions were compared with *myc* family (C-, L-, and N-*myc*) gene status obtained from a previous study,[13] we could not observe a significant correlation between Bcl_2 overexpression ($P = 0.123$) or its ratio to Bax ($P = 0.1569$) and *myc* DNA amplification and/or mRNA overexpression.

p53, Bax, and Bcl_2 Expression in Relation to Clinicopathological Parameters

p53, Bax, and Bcl_2 profiles of expression were clearly related to the histological class of NE tumors. They distinguished carcinoids (TCs and ACs) from the group of the two others (LCNECs and SCLCs). As only five ACs could be included in the study, there was no definitive conclusion as to their distinction from TCs. There was no correlation, along the spectrum of NE tumors, between p53 immunophenotype and the extension stage. However, despite the absence of a significant relation between Bcl_2 or Bax expression with tumor stage, we found a significant relation of Bcl_2:Bax ratio with tumor stage when SCLCs were excluded (almost all at extensive stages III to IV). Of 26 NE tumors with Bcl_2:Bax ratio >1, only 12 (46%) were at stage I-II, whereas of 24 NE tumors with Bcl_2:Bax ratio ≤1, 18 (75%) were at stage I or II ($P = 0.0475$).

p53 immunophenotype had no statistical influence on survival inside the group of NE tumors excluding SCLCs (TCs, ACs, and LCNECs). In contrast, a high level of Bcl_2 expression correlated with shorter survival ($P = 0.004$; Figure 2A) and a high level of Bax expression correlated with longer survival ($P = 0.0024$; Figure 2B) in a Kaplan-Meier analysis of this population (log-rank Mantel-Cox statistical analysis). As well, Bcl_2:Bax ratio ≤1 correlated with longer survival ($P = 0.0052$; Figure 2C) in a univariate analysis. However, no significant influence of Bax, Bcl_2, or their ratio could be demonstrated when TCs were excluded (in the group of ACs and LCNECs), probably due to insufficient size of the population studied.

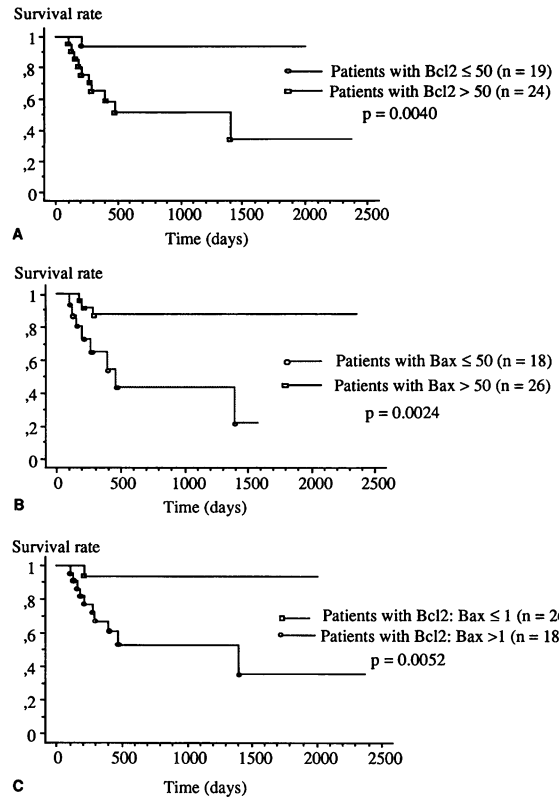

Figure 2. *Cumulative survival Kaplan-Meier analysis curves for patients with carcinoid (TC and AC) and LCNEC. Scores represent percentage of stained cells × intensity (1 to 3).* **A:** *Comparing patients with Bcl_2 score ≤ or >50 (P = 0.0040).* **B:** *Comparing patients with Bax scores ≤ or >50 (P = 0.0024).* **C:** *Comparing patients with Bcl_2:Bax score ratio ≤ or >1 (P = 0.0052).*

In a multivariate analysis (Mantel-Cox) including stage, age, histology, and Bcl_2:Bax ratio, only histology influenced the prognosis ($P = 0.0175$).

We could not demonstrate any influence of Bcl_2 or Bax levels or their ratio on an objective response to drugs or the type of response in 50 patients treated for SCLC who were evaluated in this study.

Discussion

Bax and Bcl_2 immunostaining were both detected in the cytoplasm in accordance with previous demonstration of their presence in the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum.[30,31] We found clearly distinctive profiles of expression of Bax and Bcl_2 between low-grade NE tumors (TCs and ACs) and high-grade NE tumors (LCNECs and SCLCs) with a predominance of Bax overexpression in the first group and of Bcl_2 in the second. The prevalence of Bcl_2 protein expression in SCLCs was previously demonstrated,[32–34] but this is the first report on the simultaneous expression of Bcl_2 and Bax in the spectrum of NE lung tumors. As

these factors regulate cell death, the present results suggest that a low rate of cell division in carcinoids is well compensated by a low rate of cell death, induced by Bax expression (effector of apoptosis) and the absence of Bcl_2 expression (dominant inhibitor of Bax). In keeping with this view, a high rate of cell death was found in a semiquantitative analysis of 52 carcinoids (TCs and ACs), in which a much higher rate of cell death (17 and 13%, respectively) was found in these NE low- and intermediate-grade tumors than in any other tumor type studied, such as squamous lung carcinoma.[35] However, although exhibiting, in our hands, variable TUNEL-based AIs, only one TC case had a high percentage of marked cells (10%).

In contrast, the effect of a high rate of cell division in LCNECs and SCLCs (with a mean Ki-67 index of 50%, data not shown) could be worsened by abrogation of cell death, provided by high Bcl_2 and low Bax levels of expression, which could all contribute to their short doubling time and aggressiveness. In favor of the hypothesis that Bcl_2-Bax levels could regulate population size and tumor progression was the correlation we found between extension of the disease at the time of diagnosis (stage status) and the Bcl_2:Bax ratio in NE tumors others than SCLCs; the latter presented almost all extensive disease and introduced a bias in the statistical evaluation. Moreover, Bcl_2 overexpression, Bax down-regulation, and Bcl_2:Bax score ratio >1 correlated with a statistically shorter survival in the group of patients with carcinoids (ACs and TCs) and LCNECs but not in each individual histological type due to insufficient population size. As these factors did not influence survival in a multivariate analysis, it appears that histological classification is a better predictor of survival than Bcl_2:Bax ratio. However, the Bcl_2:Bax status may be useful to predict clinical behavior in NE non-small-cell lung tumors, the differential diagnosis of which is somewhat difficult. Low Bax immunoreactivity correlated with low AI, as determined by TUNEL, in both LCNEC and SCLC subgroups; high Bcl_2:Bax score ratios were correlated with low AIs in the LCNEC subgroup and in the pool LCNECs plus SCLCs. Even when the inverse correlation Bcl_2-TUNEL was not apparent in SCLCs, Bcl_2-TUNEL double labelings indicated that tumor areas expressing Bcl_2 did not contain cells with apoptotic morphology and DNA fragmentation. In the present series of NE tumors, p53 did not correlate with extension stage, in contrast with previous results on NSCLCs,[12,36] and had no influence on survival in NE lung tumors.

Bcl_2 expression was previously shown to regulate development of normal and neoplastic cells of the sympathetic autonomic nervous system, Bcl_2 being down-regulated during final terminal differentiation.[37] In untreated neuroblastoma, two studies lead to contradictory results; one correlated the Bcl_2 overexpression with tumor progression, poor prognosis, and unfavorable histology,[21] whereas another associated Bcl_2 expression with favorable histology.[37] Our results on NE lung tumors are in accordance with the first of these studies on neuroblastoma.[21] Using the same monoclonal antibody, with a cut-off at 25% of positive cells very comparable to the one we used, both studies demonstrated a correlation between Bcl_2 overexpression and aggressiveness in these malignant NE tumor types.

As previously shown in neuroblastoma,[21,37] we found that Bcl_2 was up-regulated after therapy as compared with pre-therapy samples. It should be noted that all post-therapy samples that we examined came from patients in a definite state of chemoresistance.[38] Although immunohistochemical analysis did not provide information on the functional status of the proteins, it is tempting to speculate that only those tumor cells that contained a high level of Bcl_2 were able to survive therapeutic intervention. Presumably, proliferation of Bcl_2-protected clones, highly resistant to chemotherapy, characterized these secondary chemoresistant tumors.

It was somewhat unsurprising that Bcl_2:Bax ratio was not correlated with primary chemoresistance in SCLCs. Indeed, carcinoids, which are well known chemoresistant tumors, expressed high Bax and low Bcl_2, which should have rendered them susceptible to apoptosis, whereas SCLCs, despite a high Bcl_2 expression, are chemosensitive tumors in primary intention. This paradox could be explained by recalling that if the sensitivity of epithelial cells to DNA-damaging agents may be regulated by the Bax-Bcl_2 level, their individual tolerance to DNA damage is to be taken into account.[39] In this regard, carcinoid tumor cells have slow proliferative rates (mean Ki-67 index = 3%), which render them radio- and chemoresistant despite high Bax expression, whereas SCLC cells are actively dividing (mean Ki-67 index = 50%), which is probably responsible for their primary chemosensitivity despite Bcl_2 protection.

As in other tissues such as lymph nodes, colon, pancreas (endocrine cells), and salivary glands,[39] a reciprocal pattern of Bax and Bcl_2 protein production was observed in most NE lung tumors. Interestingly, both normal NE cells and basal stem cells, which are candidates for NE cell proliferation, overexpress Bcl_2, which could allow the maintenance of their functions (chemoreception, neurotransmission, and regeneration). The Bcl_2 expression was selected

only by their more malignant clones, in addition to other genetic alterations.

p53 was found to regulate the Bax-Bcl_2 balance, and wild-type p53 function may be required for the optimal expression of Bax under the form of a Bax-Bax dimer, in some cell types at least. p53 mutation, in these cell types, would result in a marked reduction of Bax and increase of Bcl_2, which seems to be the case in NE lung tumors. In those tumors with an apparently normal p53 phenotype, such as TCs, Bcl_2-Bax balance was in favor of susceptibility to apoptosis. Moreover, previous studies have demonstrated that p53 was in its wild-type form in carcinoids.[40] In contrast, LCNECs and SCLCs showed the same high frequency of mutant p53 immunophenotype and a characteristic Bcl_2:Bax ratio >1. This is in keeping with a dose-dependent regulation of the Bcl_2-Bax balance by p53 in malignant NE cells.

However, almost 50% of tumors with negative p53 immunostaining overexpressed Bcl_2. There are two alternative or complementary explanations. First, p53-negative immunostaining is much less tightly associated with p53 wild-type function than is p53-positive immunophenotype with mutation. We previously showed[13] that a number of mutations abolish normal transcription and normal translation leading to null p53 phenotype (no p53 protein). These and some nonstabilizing point mutations allow discrepancies between negative immunophenotype and p53 status, which accounted for approximately 20% of false negatives using immunostaining. This could explain the deregulation of Bcl_2:Bax ratio in some of the p53-negative cases. The second and very probable explanation is that *bcl_2* could be primarily and constitutively deregulated by somatic genetic or epigenetic events in aggressive NE lung tumors. Furthermore, the absence of correlation between TUNEL and p53 status in lung NE tumors may be explained by the indirect and incomplete, Bcl_2-Bax-mediated control of active cell death by p53. Bcl_2 blocks p53-dependent apoptosis, and clones with Bcl_2 deregulation could be selected in highly malignant tumors that have retained wild-type p53 functions.[41] The ability of Bcl_2 to bypass induction of apoptosis by p53 may contribute to its oncogenic and anti-apoptotic activity in LCNECs and SCLCs. Thus, Bcl_2-Bax imbalance could be both p53 independent and p53 dependent (exaggerated by p53 mutation) in highly malignant NE tumors. Cooperation between p53-dependent and p53-independent apoptotic pathways have been demonstrated, and mutant p53 can block both pathways.[42] Bcl_2 could be attenuated in these tumors or Bax up-regulated, which might provide therapeutic approaches. Moreover, Bcl_2 has been shown to interact with other members of the Bcl_2 family that are Bcl_2 homologues, such as Bcl-x_S and Bcl-x_L, as well as non-Bcl_2-like proteins including Bag1, Raf1, and R-Ras.[43]

LCNECs had not been previously studied at a molecular level. We showed here that they have more similarities with SCLCs than believed from their large-cell morphology. As SCLCs, they displayed NE differentiation high rate of proliferation, high expression of Bcl_2, and frequent p53 mutation. They should thus be considered in the future on the same basis as SCLCs with regard to therapeutic intervention. However, LCNECs display an incidence of apoptosis significantly higher than that of SCLCs (Table 5), and this may influence their response to therapy.

As Bcl_2 has been shown to block Myc-dependent apoptosis in tumors, it is interesting to note that, of 40 cases of the present series previously studied for C-, L-, and N-*myc* amplification and/or overexpression,[9,13] 13 cases displayed *myc* amplification and/or mRNA overexpression without any significant correlation between *myc* amplification and/or overexpression and *bcl_2* level of expression. This corroborates previous results on neuroblastoma[37] where Bcl_2 overexpression was dissociated from N-*myc* overexpression.

Bcl_2 expression and its p53 partial dependence in NE lung tumors are inverse to that observed in non-NE lung tumors (non-SCLCs),[22,23] where an inverse relationship was found between Bcl_2- and p53-positive immunostaining. Moreover, survival probability was higher in patients who expressed Bcl_2 protein. It should be noted that Bax was not evaluated in these studies and that both studies used a cut-off of 1% for positive Bcl_2, which is different from the cut-off used in our semiquantitative evaluation. However, differences probably exist between NE and non-NE populations and reside in the selectivity and tissue specificity of Bcl_2-Bax expressions and other members of their family, which presumably regulate NE and non-NE cells in different ways.

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